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Energy-Yielding Properties of SoxB-Type Cytochrome bo_3 Terminal Oxidase: Analyses Involving *Bacillus stearothermophilus* K1041 and Its Mutant Strains[1]

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We isolated a K17q8 mutant from K17 mutant cells of *Bacillus stearothermophilus* which contain SoxB-type cytochrome bo_3 as well as cytochrome *bd* but not SoxM-type cytochrome caa_3, which is the main terminal oxidase in *B. stearothermophilus* K1041. The respiration of K17q8 was highly sensitive to as little as 10 μM cyanide, indicating that the main terminal oxidase is cytochrome bo_3. The aerobic growth yield of K17q8 was lower than that of wild-type K1041, but higher than that of parental K17. The H^+/O ratio of K17q8 was about 5, *i.e.* a little lower than the 6.1–6.5 of K1041, but higher than the 2.9–3.1 of K17 [Sone *et al.* (1999) *J. Biosci. Bioeng.* 87, 495–499]. Analyses of membrane fragments indicated that K17q8 contains about 0.2 nmol cytochrome bo_3 per mg membrane protein, and scarcely any subunits of cytochromes caa_3 and *bd*. From the membrane fraction of K17q8, cytochrome bo_3 was purified and shown to be composed of two subunits with apparent molecular masses of 56 and 19 kDa. The enzyme contained protoheme IX and heme O, as the main low-spin heme and high-spin heme. Analysis of the substrate specificity indicated that the high-affinity site is very specific to cytochrome *c*-551, a cytochrome *c* which is a membrane-bound lipoprotein of thermophilic *Bacillus*. The I_{50} of purified cytochrome bo_3 was determined to be 4 μM, indicating that cytochrome bo_3 among the three terminal oxidases in *B. stearothermophilus* was most susceptible to cyanide. The respiration of K17q8 was mostly inhibited by the addition of cyanide at this concentration.

Key words: *Bacillus stearothermophilus*, cytochrome *c* oxidase, growth yield, H^+/O ratio, SoxB-type heme-copper oxidase.

Most of the terminal oxidases in the respiratory chains belong to the heme-copper oxidase family (*1*, *2*), which can be classified into three subfamilies, *i.e.* the SoxM-, SoxB-, and FixN-types, based on the structures of their subunits I and II (*2*, *3*). The majority of oxidases including mitochondrial ones are of the SoxM-type, and their subunit II contains two transmembrane helices. SoxB-type oxidases, which have a single transmembrane helix in their subunit II, have been cloned from archaebacteria (*4*, *5*), *Thermus thermophilus* (*6*), and *Bacillus stearothermophilus* (*7*). FixN-type oxidases are cytochromes cbb_3, which do not contain any proteins similar to subunit II of the other oxidase types (*2*, *3*).

The main terminal oxidase of thermophilic Gram-positive bacilli such as *Bacillus* PS3 and *B. stearothermophilus* is a SoxM-type cytochrome caa_3, at least under highly-aerated conditions (*8*), which has a built-in cytochrome *c* moiety fused to subunit II (*9*, *10*). We recently obtained a *B. stearothermophilus* mutant devoid of caa_3-type oxidase (*11*). This mutant, K17, shows very low TMPD oxidase activity and a very low heme A content, but still actively respires through two alternative oxidases; cytochrome *bd*-type quinol oxidase, composed of two subunits of 52 and 40 kDa (*11*, *12*), and $b(o/a)_3$-type cytochrome *c* oxidase, composed of 56- and 19-kDa subunits (*13*). The former enzyme, playing a role as the major terminal oxidase in K17 (*11*, *12*), is homologous to cytochrome *bd* from proteobacteria (*14*, *15*). The latter enzyme, operating as the minor oxidase in K17, was purified and shown to specifically oxidize cytochrome *c*-551 (*16*, *17*) of this bacterium (*12*). Sequencing of the gene for this enzyme (*7*) revealed that it is a homologue of SoxB-type *T. thermophilus* cytochrome ba_3 (*6*). SoxB-type oxidases are different from usual SoxM-type cytochrome *c* oxidases in the lack of the so-called D channel structure for H^+ translocation in subunit I, while amino acid residues for metal binding as chromophores are the same as in SoxM-type enzymes (*7*, see also Ref. *18* for a review). Recently, *T. thermophilus* ba_3-type oxidase was shown to oxidize cytochrome *c*-552 (*19*, *20*) of this bacterium with concomitant

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Abbreviations: CCCP, carbonylcyanide *m*-chlorophenyl-hydrazone; HOQNO, 1-heptyl,4-hydroxyquinoline *N*-oxide; kDa, kilodalton; MEGA 9 + 10, 1:1 mixture of *n*-nonanoyl *N*-methylglucamide and *n*-decanoyl *N*-methylglucamide; NTG, 1-methyl-3-nitro-1-nitrosoguanidine; SDS-PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

H^+ translocation, although the efficiency of H^+ pumping was lower than that of SoxM-type enzymes (*21*). Since it was rather difficult to obtain $b(o/a)_3$-type oxidase from either the wild-type *B. stearothermophilus* or K17 mutant cells, because of its low content, we tried to obtain a mutant in which this enzyme operates as the major terminal oxidase. The K17q8 strain, derived from K17, was inhibited by a very low concentration of cyanide, suggesting that SoxB-type cytochrome $b(o/a)_3$ is the main terminal oxidase able to change the energy-yielding properties of cells.

Here we report the cytochrome patterns, and electron-transferring and energy-yielding properties of this K17q8 mutant in comparison with those of the parental strains. In K17q8 cells, NADH seems to be oxidized through NADH dehydrogenase, menaquinol, the cytochrome b_6c_1 complex, cytochrome *c*-551, and $b(o/a)_3$-type cytochrome *c* oxidase.

MATERIALS AND METHODS

Materials—MEGA 9 and MEGA 10 were purchased from Dojin (Kumamoto). DEAE-Toyopearl anion exchange gel and hydroxyapatite were obtained from Tosoh (Tokyo) and Bio-Rad (Hercules), respectively. The proteins used as molecular mass standards and cytochromes *c* of yeast and bovine heart were purchased from Sigma Chemicals (St. Louis). Cytochrome *c*-551 of *Bacillus* PS3 was overexpressed in *B. stearothemophilus* K1041 and prepared as described previously (*22*). TMPD, rotenone, abietic acid and HOQNO were purchased from Wako Pure Chemicals (Osaka). Lysozyme, *p*-benzoquinone, and DNase were purchased from Seikagaku Kogyo (Tokyo), Sigma Chemicals (St. Louis), and Boehringer (Mannheim), respectively. Other reagents were of analytical grade.

Strains and Culturing of the Cells—A *B. stearothermophilus* mutant, strain K17q8, was isolated by mutation and selection from K17 (*11*) as follows: The K17 strain was firstly treated with 0.1 mg/ml of NTG for 20 min at 10°C. After washing three times with the culture medium, the cells were grown in the medium containing 0.02% *p*-benzoquinone for 12 h. An 10 μl aliquot of the culture medium was spread on a plate after dilution. Ten colonies out of about 200 colonies were cultured in test tubes, the oxygen uptake and cyanide-sensitivity being monitored. Although the respiration of most of these cells was more or less inhibited by 0.01 mM NaCN, a strain showing 80% inhibition was selected and named K17q8. These mutant strains and the wild-type strain K1041 (*21*) were cultured at 60°C in the medium containing 0.8% polypeptone, 0.1% yeast extract, 0.3% NaCl, and 0.1% dipotassium phosphate, with the pH adjusted to 7.0–7.2. The preculture was carried out overnight in a test tube, and the bacteria were grown in a baffled flask (1 liter) containing 200-ml medium shaken rather vigorously (180 times/min) after inoculation (1%). The ab-sorbance was monitored at 650 nm after 10-times dilution to follow the growth of the cells. Harvesting, washing and preparation of the membrane fragments were carried out as described previously (*23*).

Purification of Cytochrome bo_3—Cytochrome bo_3 was prepared from K17q8 cells as described previously for purification of the enzyme from K17 (*13*).

Measurement of Oxidase Activity and Optical Spectra—The respiration of cells and oxygen uptake by membranes were measured using a Clark-type oxygen electrode in a reaction medium (2.4 ml) composed of 100 mM NaCl, 1 mM EDTA, and 50 mM sodium phosphate buffer, pH 6.0, at 45°C (*13*). The cytochrome *c* oxidase activity of the purified enzyme was continuously monitored with a pH electrode (Beckman No. 39030) as described elsewhere (*10*). The standard reaction medium contained 15 nM $b(o/a)_3$-type oxidase, 0.4 μM cytochrome *c*-551, 125 μM TMPD, 5 mM Na- ascorbate, 1 mM $MgSO_4$, 150 mM KCl, and 1 mM Na-P_i buffer, pH 6.7. The reaction was initiated by adding cytochrome *c*-551 of *Bacillus* PS3 to 2.5 ml of the reaction mixture containing the other constituents at 40°C with stirring. The turnover number was expressed as e^-/enzyme/s.

Absorption spectra were obtained with a Beckman DU-70 spectrophotometer. The spectra of air-oxidized forms were taken and then a few grains of solid sodium dithionite were added to obtain the reduced forms. The contents of cytochromes aa_3 and *c*-551 were determined from the reduced *minus* oxidized difference spectra using millimolar extinction coefficient differences of 21.3 at 604–630 nm (*7*), and 24.3 at 551 nm (*16*), respectively. The contents of cytochromes bo_3 and *bd* were determined from the CO-reduced *minus* reduced difference spectra using millimolar extinction coefficient differences of 206 at 416–430 nm (Mogi, unpublished result for *E. coli* bo_3), and 23.5 at 625–650 nm (*13*), respectively.

Other Analyses—For measurement of the H^+/O ratio, the pH change induced by an oxygen pulse with resting cells under anaerobic conditions was measured as described previously (*23*). The solubility of O_2 in 0.14 M KCl was estimated to be 0.36 μg-atom O/ml at 45°C. Protein determination and SDS-PAGE were carried out as previously (*12*), and the gel was stained for protein and heme (*27*).

RESULTS

Isolation of a B. stearothermophilus Mutant Using SoxB-Type Cytochrome bo_3—In order to select a mutant having SoxB-type cytochrome $b(o/a)_3$ as the main terminal oxidase, we used the K17 mutant of *B. stearothermophilus* as the parental strain. K17 cells only oxidize TMPD very slowly, since quinol-oxidizing cytochrome *bd* shows very low TMPD oxidase activity (*11*, *23*) and the content of cytochrome *c*/TMPD-oxidizing cytochrome $b(o/a)_3$ is low. We tried to isolate a mutant strain by treating K17 cells with NTG and selecting a colony which turned purple upon TMPD addition. Such a mutant dominantly expressed should oxidize TMPD rather rapidly. However, we could not find such colonies, probably because the TMPD-oxidizing activity of cytochrome $b(o/a)_3$ is not sufficient in comparison to the activity needed to reduce it, even if the amount of cytochrome $b(o/a)_3$ in the mutant is increased several-fold. Then we tried to isolate a strain that grows aerobically even in the presence of *p*-benzoquinone, which inhibits quinol oxidases such as *E. coli* cytochrome *bo* (*24*) and *B. stearothermophilus* cytochrome *bd* (*12*). The parental K17 strain was able to grow in the presence of 0.01% *p*-benzoquinone, but not in the presence of 0.02% or higher. NTG-treated K17 cells were cultured in the liquid medium containing 0.02% *p*-benzoquinone, and a part of the liquid medium was poured onto a plate to isolate colonies. One colony whose endogenous respiration was inhibited to 20% on the addition of 0.01 mM NaCN was chosen as a candidate mutant having cytochrome $b(o/a)_3$ as the main termi-

nal oxidase, and named K17q8. Cytochrome $b(o/a)_3$ was shown to be inhibited by cyanide at as low a concentration as 0.01 mM (*13, 23*).

Growth Curve and H^+/O Ratio of the Mutant—Figure 1 shows the growth curve of K17q8 in comparison with those of the parental strains, K1041 and K17. The cell yield of K17q8 at the stationary phase was greater than that of K17, and smaller than that of K1041. The doubling time of the new mutant was almost the same as that of the wild type under the culture conditions used.

In order to determine the energetic basis for the difference in the growth yield, we measured the H^+/O ratio by the oxygen pulse method and evaluated the efficiency of the respiratory chain (*8, 26*). Figure 2 shows typical pH change results upon oxygen pulse with resting K17q8 cells in the presence of the permeant anion SCN- and valinomycin plus K^+. The observed H^+/O ratio was 4.9 (mean of 5 experiments), which is lower than that of the wild-type cells, 6.4, but higher than that of the K17 mutant cells, 3.1 (*23*). In the presence of TMPD, the apparent H^+/O ratio was 2.1, indicating that the net H^+/O ratio was 1.1, which is almost half of that of SoxM-type enzymes.

Effects of Cyanide and HOQNO on the Oxidase Activity—Figure 3 shows the effects of the cyanide concentration on the respiration of K17q8 and wild-type cells. The endogenous respiration of the wild type cells was inhibited biphasically, as observed previously (*23*), indicating that an alternative quinol oxidase with low cyanide-sensitivity, probably cytochrome *bd*, operates when caa_3-type cytochrome *c* oxidase is inhibited. On the other hand, 70% of the endogenous respiration of K17q8 was inhibited on the addition of cyanide at a very low concentration, *i.e.* below 0.01 mM (I_{50} = 4 μM). Partial inhibition by cyanide in this range was observed in K17 cells, but it was less than 15% (*23*). It is thus likely that $b(o/a)_3$-type oxidase operates in K17q8 as the main terminal oxidase, whereas this enzyme acts as an auxiliary oxidase besides the main *bd*-type quinol oxidase in K17.

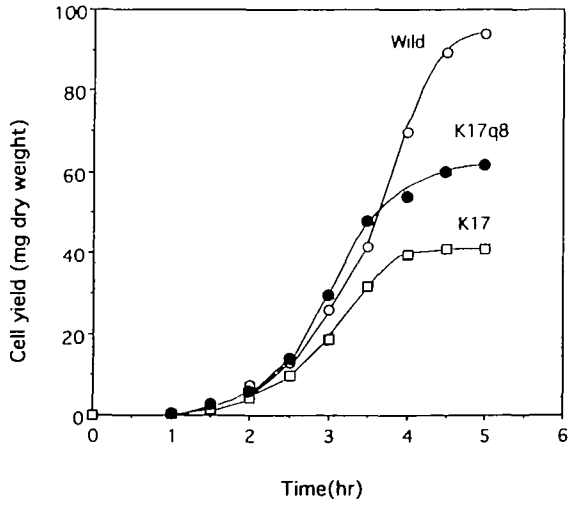

Fig. 1. **Growth curves of *B. stearothermophilus* and its mutant strains.** The growth conditions are given under "MATERIALS AND METHODS." ○, K1041 (wild-type); □, K-17; ●, K17q8. Cell yields are expressed in dry weight for a 100 ml–scale culture in a 500 ml baffled flask.

HOQNO is a wide-range inhibitor of bacterial quinol-cytochrome *c* reductase (*25, 27*), while antimycin A inhibits the mitochondrial/purple bacterial quinol-cytochrome *c* reductase (the bc_1 complex) potently (*28*). Figure 4 shows the inhibition by HOQNO of NADH oxidation by K17q8 membranes in comparison with in the cases of the wild-type K1041 and K17 membranes. The oxidation by K17q8 was largely inhibited by a low concentration of HOQNO, like the oxidation by K1041 was, while the oxidation by K17 was inhibited only slightly. Since the I_{50} value of HOQNO of the purified quinol-cytochrome *c* reductase of *B. stearothermophilus* is about 1 μM (*27*), NADH oxidation by

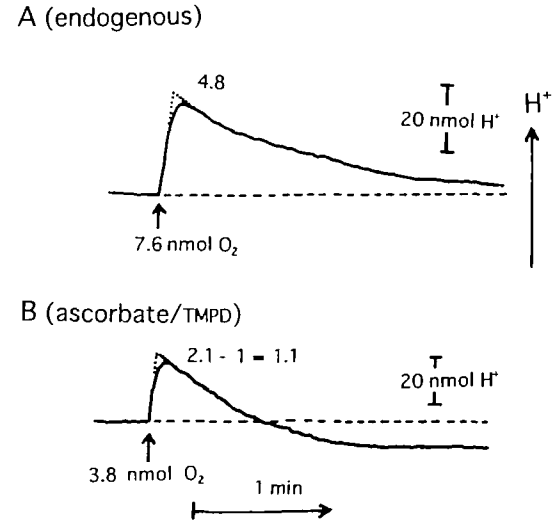

Fig. 2. **pH changes upon oxygen pulse of anaerobic cell suspensions of resting K17q8 cells.** Cells (3.7 mg dry wt.) were incubated at 45°C in 3.2 ml of 140 mM KCl containing 50 mM KSCN and 0.25 mM K-Mops (pH 6.3–6.5). After anaerobiosis, 0.5 μg valinomycin was added, and after about 5 min the reaction was started by adding air-saturated 0.14 M KCl (20 μl containing 7.6 ng-atomic O). The buffer action of the medium was determined by titration with a 2 μl aliquot of 10 mM HCl, as indicated by bars. A, endogenous respiration; B, TMPD oxidation in the presence of 10 μM HOQNO, 0.1 mM TMPD, and 5 mM ascorbate.

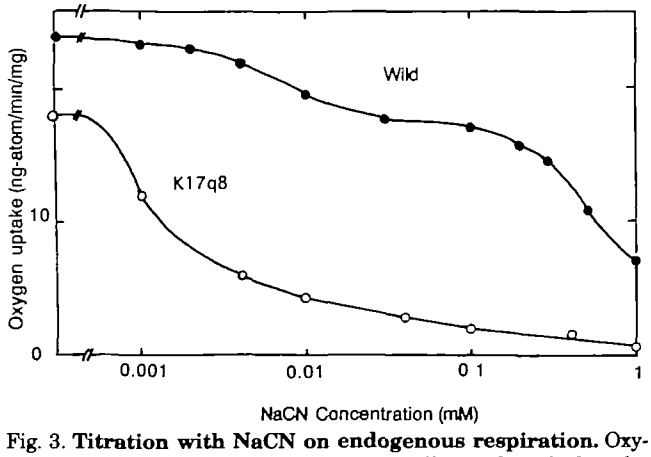

Fig. 3. **Titration with NaCN on endogenous respiration.** Oxygen uptake was monitored polarographically as described under "MATERIALS AND METHODS." The rate of oxygen uptake was measured at 5 min after the addition of NaCN to give the indicated concentration. ○, K17q8 cells (1.50 mg dry wt.); ●, K1041 (wild-type) cells (1.26 mg dry wt.).

K17q8 is suggested to occur *via* quinol-cytochrome *c* reductase. Taken together, it is concluded that electron flows *via* NADH dehydrogenase, menaquinone, quinol-cytochrome *c* reductase, cytochrome *c*-551, and cytochrome bo_3 in K17q8 membranes. The small difference in the degree of inhibition by K17q8 and K1041 is probably due to the different degrees oxidation through unidentified route(s) other than quinol-cytochrome *c* reductase. Since the percentage inhibition by 10 μM HOQNO of K17q8 was lower than that in the case of K1041, a portion of the oxidation by K17q8 may occur without the use of quinol-cytochrome *c* reductase. On the contrary, the respiration of K17 was scarcely inhibited by a low concentration (3 μM) of HOQNO, and the weak inhibitory effect may have been caused by the HOQNO inhibition of the *bd*-type quinol oxidase, since the I_{50} of *E. coli* cytochrome *bd* was reported to be 7 μM (*25*). A different inhibition pattern of K17q8 from that of K17 was also observable with *p*-benzoquinone. This inhibitor at a very low concentration inhibited the endogenous respiration of K17, since the *bd*-type quinol oxidase was very susceptible to this inhibitor (*23*), while K17q8 respiration was rather resistant (not shown).

Constituents of the Respiratory Chain of K17q8—The redox difference spectrum of the K17q8 membranes (Fig. 5B) showed the alpha peak at 560 nm like that of the membranes from K17 (Fig. 5A). The small difference in the shape of the 550–560 nm region may be partly caused by the amounts of some *c*-type cytochromes such as cytochrome *c*-551 (*16*) and cytochrome b_6c_1 of quinol-cytochrome *c* reductase (*27*), as well as the amount of cytochrome bo_3, whose spectrum is shown in Fig. 5C. The K17q8 membrane contains more cytochrome bo_3 than the K17 membrane does.

The difference absorption spectrum of CO-reduced *minus* reduced forms of the solubilized membranes from K17q8 (Fig. 6B) shows peaks at 572, 537, and 415 nm, and troughs at 563 and 432 nm, indicating that CO binds to heme O. There was no indication of the presence of another heme such as hemes A and D other than heme O at the high-spin heme site of the binuclear center, suggesting that cytochrome bo_3 is the solo terminal oxidase. We previously found heterogeneity of high-spin heme in cytochrome $b(o/a)_3$ isolated from K17 (*13*). The air-limited conditions of the present culture may prevent ctaA (heme O oxygenase/oxidase) from producing heme A, since similar conversion of the high spin heme of the binuclear center from heme A to heme O takes place in cytochrome caa_3 of thermophilic

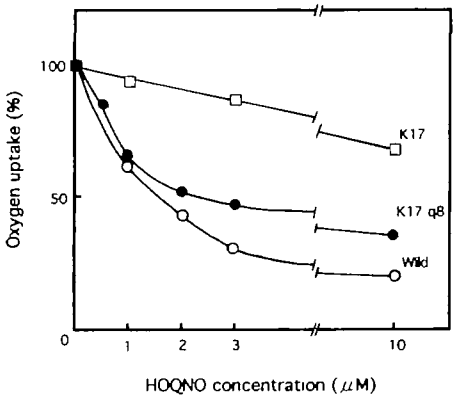

Fig. 4. **Titration with HOQNO on NADH oxidation of membranes from three different strains.** Oxygen uptake was monitored polarographically as described under "MATERIALS AND METHODS." The reaction was started by the addition of NADH (final concentration, 0.4 mM). The rate of oxygen uptake was measured 5 min after the addition of HOQNO at the indicated concentration. ○, K1041 membrane (0.19 mg protein); □, K17 membrane (0.49 mg protein); ●, K17q8 membrane (0.17 mg protein).

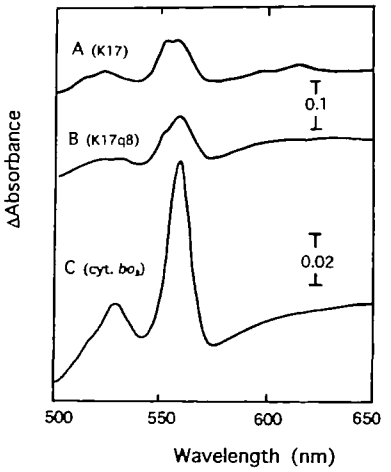

Fig. 5. **Redox difference spectra of membranes from the mutant strains of *B. stearothermophilus*.** $Na_2S_2O_4$-reduced *minus* oxidized (as prepared). A, solubilized membranes from K17 (7.2 mg/ml); B, those from K17q8 (7.3 mg/ml); C, the purified cytochrome bo_3 from K17q8 (3.1 μM).

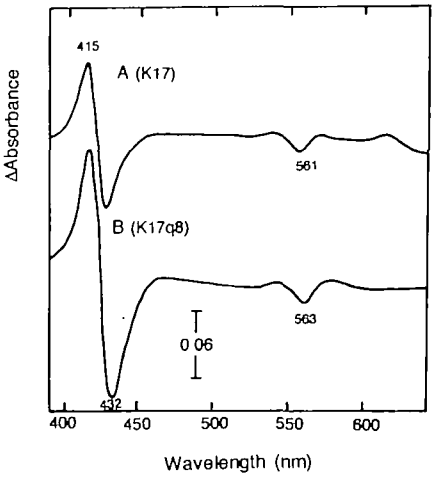

Fig. 6. **CO-difference spectra of solubilized membranes from the mutant strains of *B. stearothermophilus* and the purified cytochrome.** A, solubilized membranes from K17 (7.2 mg/ml); B, those from K17q8 (7.3 mg/ml).

TABLE I. **Oxidase activities and terminal oxidase contents of *B. stearothermophilus* and its mutants.**

Strain	Oxidase activity (ng atom/mg/min)		Terminal oxidase (nmol/mg)		
	NADH	TMPD	cyt. aa_3	cyt. *bd*	cyt. bo_3
K1041, wild	539	353	0.21	0.02	n.d
K17	585	34	n.d	0.12	<0.08
K17q8	545	128	n.d	n.d	0.18

n.d, not detectable.

Bacillus PS3 (*29*). We call the present SoxB-type cytochrome *c*-551 oxidase cytochrome bo_3. The spectrum of K17 (Fig. 6A) shows an apparent peak at 620–625 nm, indicating the presence of cytochrome *bd*.

Table I summarizes the oxidase activities and cytochrome contents reacting with O_2 of membrane fractions from the three different strains. Each preparation showed high NADH oxidase activity, respectively, while their TMPD oxidase activities were different. The membranes from K1041 showed the highest activity, the K17 membranes the lowest, and the K17q8 membranes an intermediate value. These differences should be caused by the different abilities of the three terminal oxidases present in the strains. Cytochrome caa_3 was observed in K1041, not observed in K17, and very low, if any, in K17q8. As to TMPD oxidase activity, cytochromes caa_3 and bo_3 in the purified form show high TMPD oxidase activity, while quinol-oxidizing cytochrome *bd* almost does not oxidize TMPD at all (*11*, *12*).

Figure 7 shows an electrophoregram of the membrane fragments of K17q8 in comparison with in the cases of K1041 and K17. A band at 42 kDa, invisible for the K17 (lane 2) and K1041 membranes (lane 3), was observed for the K17q8 membranes (lane 1), although the presence of another band at a little lower position makes the fact unclear in lane 3. Subunit I of the purified cytochrome bo_3 (lane 4) appeared at the same position. Detection of subunit II of cytochromes caa_3 (38 kDa) and cytochrome c_1 (29 kDa), and cytochrome *b* subunits (21 kDa) of the b_6c_1 complex is possible by heme-staining (*7*, *27*), and these bands were successfully detected for K1041 membranes (lane 7). Subunit II of SoxM-type oxidase was hardly detectable in the K17q8 (lane 5) and K17 (lane 6) membranes. On the other hand, cytochrome c_1 (29 kDa) and its proteolytic product (having heme C, around 23 kDa) were more or less present in all membranes, while cytochrome *b* (21 kDa) was clearly observed in lanes 5 and 7, but not in lane 6 for K17 membranes.

Purification of Cytochrome bo_3 and Its Properties—In order to confirm the presence of cytochrome bo_3, we purified cytochrome bo_3 from K17q8, according to the previously described method for purification from K17 (*13*). After the purification on DEAE-Toyopearl and hydroxyapatite columns, the sample was almost pure, as judged on SDS-PAGE (lane 4 in Fig. 7), although the band due to subunit II was obscure. It has been reported that this protein was not stained well by Coomassie Brilliant Blue (*13*). About 50 nmol cytochrome bo_3 was isolated from 5 g wet cells (2 liter-scale culture). The redox difference spectrum was very similar to that of the enzyme from K17 (*12*). The CO difference spectrum of the purified enzyme from K17q8 (not shown) was very similar to that shown in Fig. 6B, indicating that cytochrome bo_3 is the sole CO-binding cytochrome pigment and heme A is scarcely present.

We previously reported that cytochrome *c*-551, at a very low concentration, highly activates the TMPD oxidase activity of cytochrome bo_3 purified from K17, especially under high ionic strength conditions (*13*). This activation followed Michaelis-Menten kinetics with a K_m of as low as 0.13 μM for cytochrome *c*-551, indicating that the high affinity site of cytochrome bo_3 was specifically occupied by *c*-551. The present cytochrome bo_3 purified from K17q8 cells oxidized TMPD at a high turnover rate (V_{max} = 130 s^{-1}) in the presence of cytochrome *c*-551 occupying the high affinity site (K_m = 0.12 μM). Among the three terminal oxidases in *B. stearothermophilus*, cytochrome bo_3 was the most susceptible to cyanide. The I_{50} of the purified cytochrome bo_3 was

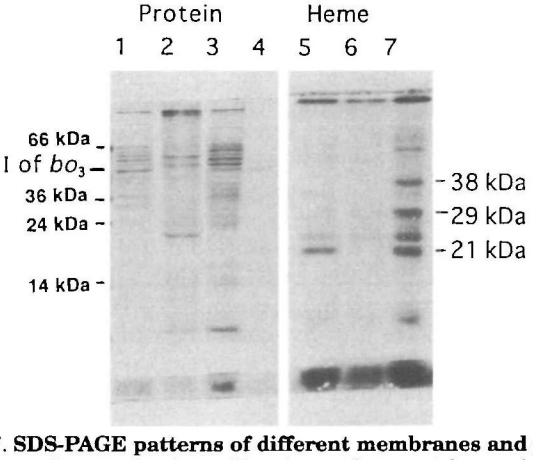

Fig. 7. **SDS-PAGE patterns of different membranes and purified cytochrome bo_3 from K17q8 membranes.** The acrylamide gel concentration was 13.5%, and Coomassie brilliant blue was used for protein staining. About 40 μg of each membrane fraction was used for the electrophoresis. Lanes 1 and 5: K17q8 membranes. Lanes 2 and 6: K17 membranes. Lanes 3 and 7: K1041 membranes. Lane 4: the purified bo_3-type oxidase (1.2 μg). Lanes 5, 6, and 7 were the same as lanes 1, 2, and 3, and were stained with *o*-tolidine and H_2O_2 for heme detection.

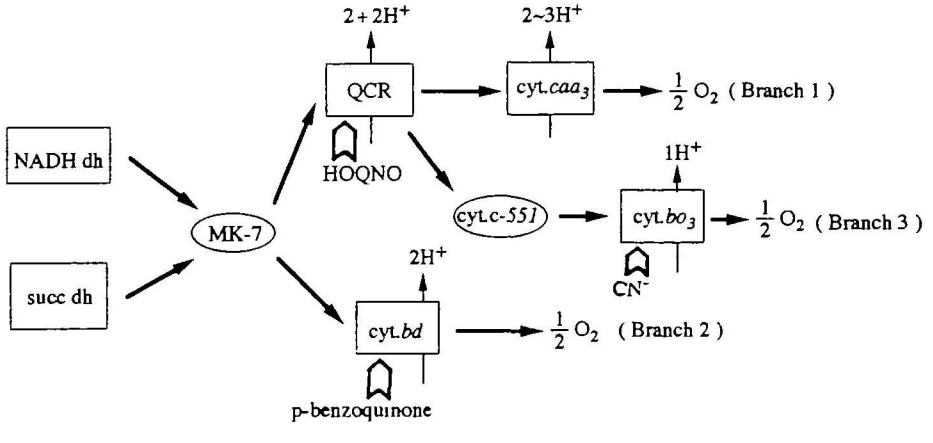

Fig. 8. **Respiratory pathways in a thermophilic *Bacillus*.** The $H^+/2e^-$ stoichiometry of each respiratory complex is tentatively indicated. Most, if not all, of NADH dehydrogenase is non-H^+ pumping (*23*). The most susceptible portions as to cyanide, *p*-benzoquinone, and HOQNO are indicated (⏃). MK-7, menaquinone-7; dh, dehydrogenase; cyt, cytochrome.

determined to be 4 μM (not shown). These kinetics with cytochrome *c*-551 and inhibition by cyanide of TMPD oxidation by the present cytochrome bo_3 preparation are very similar to the data for the enzyme from K17, as reported previously (*13*). This cytochrome *c*-551–dependent TMPD oxidation had another characteristics, it is greatly stimulated by a high ionic strength of the medium, as reported previously (*13*).

DISCUSSION

We obtained a unique mutant, K17q8, which uses SoxB-type bo_3 as the main terminal oxidase, *i.e.* as much as 0.18 nmol/mg membrane protein. To isolate this mutant, the following different characteristics as to inhibitor sensitivity of the two terminal oxidases were successfully used; *bd*-type quinol oxidase is susceptible to *p*-benzoquinone (*12*), and SoxB-type cytochrome bo_3 is inhibitable with a very low concentration of cyanide (*13*, *23*). This K17q8 mutant shows an H^+/O ratio of about 5 (Fig. 2), *i.e.* slightly lower than that of K1041, the parental wild-type *B. stearothermophilus*, but higher than that of K17, which mainly uses the *bd*-type quinol oxidase (*23*). The cell yield of K17q8 was between those of K1041 and K17 (Fig. 1). The cell yield should be dependent on the H^+/O ratio, when the H^+/ATP ratio of ATP synthase is constant. The fact that the H^+/O ratio of K17q8 was lower than that of K1041 could be explained by that either the H^+/O ratio of cytochrome bo_3 is lower than that of cytochrome caa_3, or that cytochrome *c*-551 is not reduced by the Q-cycling cytochrome b_6c_1 complex (*27*, *28*) in K17q8. The former possibility is favored by the facts that the H^+/O ratio coupled to TMPD oxidation of K17q8 is lower than that of K1041 (Fig. 2B), and that the NADH oxidation of K17q8 is inhibited by HOQNO, as that of K1041 is (Fig. 4). It is also noteworthy that *T. thermophilus* cytochrome ba_3 pumps H^+, but less efficiently (*21*). Thus at least qualitatively, K17q8 oxidizes NADH through NADH dehydrogenase, menaquinol, the cytochrome b_6c_1 complex, cytochrome *c*-551, and bo_3-type cytochrome *c* oxidase.

Figure 8 shows the respiratory chain of *B. stearothermophilus*, which contains at least three terminal oxidases; caa_3-type cytochrome *c* oxidase, *bd*-type quinol oxidase, and bo_3-type cytochrome *c*-551 oxidase. Corresponding to these three terminal oxidases, there might be three branches for the oxidization of menaquinol. Branch 1 operates in the wild type cells and should show a H^+/O ratio of over 6, if the cytochrome b_6c_1 complex and caa_3-type cytochrome *c* oxidase translocate $4H^+/2e^-$ and 2-3 $H^+/2e^-$, respectively, which explains most part of the experimentally obtained H^+/O ratio for the wild type cells. Branch 2 (*bd*-type quinol oxidase) operates in K-17 as the main pathway, as well as in the wild-type cells as a relatively CN-tolerant minor pathway. The H^+/O ratio of this branch should be 2.0, because *bd*-type oxidase has been shown only to translocate chemical protons, *i.e.* it does not pump vectorial protons, in *E. coli* (*17*). Branch 3, composed of the cytochrome b_6c_1 complex, cytochrome *c*-551 and bo_3-type cytochrome *c* oxidase, operates in K17q8 as the main pathway, and also operates in K17 as an alternative O_2 sink besides Branch 2. The H^+/O ratio of K17q8 cells was about 5, indicating that bo_3-type cytochrome oxidase is an H^+-pump, but suggesting that its efficiency is lower than that of the SoxM-type enzyme. It was of interest to determine the H^+/O ratio of cytochrome bo_3, since the amino acid sequence deduced from DNA indicates that several residues of $b(o/a)_3$ subunit I, claimed to be important for SoxM-type oxidases to translocate H^+ across the membrane, are not conserved (*7*). The obtained H^+/O ratio was about half of the value usually observed for SoxM-type oxidases, if quinol-cytochrome *c* reductase translocates $4H^+/2e^-$.

The physiological meaning of the SoxB-type alternative oxidase remains to be determined. Three types of alternative oxidases are known to operate under microaerobic conditions; cytochrome *bd*, cytochrome cbb_3, and SoxB-type oxidase. Cytochrome *bd*-type quinol oxidase can utilize O_2 at very low concentrations in many eubacteria, but does not pump H^+ (*14*). Alpha proteobacteria such as rhizobia and photosynthetic bacteria and *Helicobacter pylori* belonging to the epsilon group contain cbb_3-type cytochrome *c* oxidase under microaerobic conditions as the alternative terminal oxidase (*1*, *2*, *30*). Only two eubacteria, both of which are thermophilic, are known to have SoxB-type cytochrome *c* oxidase, while several aerophilic archaebacteria contain SoxB-type quinol oxidase (*4*, *5*). It is tempting to speculate that thermophiles, which are apt to dwell under microaerobic conditions due to the low O_2 solubility, retain the ability to biosynthesize SoxB-type cytochrome *c* oxidase with a very low K_m for O_2, which oxidizes cytochrome *c* with concomitant H^+ translocation, even if its efficiency is lower than that of the SoxM-type. We found that the K_m of the present enzyme is below 0.5 μM, as for the *H. pylori* cbb_3-type oxidase (*30*). At least K17q8 is able to grow well, indicating that the respiratory chain with cytochrome bo_3 works well under the present culture conditions.

Another interesting point about this enzyme is that the subunit I amino acid sequences of SoxB-type oxidases are different from those of SoxM-type enzymes, whose amino acid residues in the hydrophobic region supposed to constitute the H^+ pathway (D and K channels) across the membranes are not conserved, whereas all amino acid residues ligating the metal centers are conserved (*7*). It is thus likely that these structural features of SoxB-type oxidases may be the structural basis of their lower H^+/O ratios.

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